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Generation of Cross-Protective Antibodies against *Plasmodium falciparum* Sequestration by Immunization with an Erythrocyte Membrane Protein 1–Duffy Binding-Like 1α Domain▽†

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The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is an important virulence factor on the surface of infected erythrocytes. Naturally acquired antibodies to PfEMP1 expressed by parasites causing severe malaria are suggested to be protective and of major interest for the development of a vaccine against severe disease. In this study, the PfEMP1 expressed by a parasite clone displaying a multiadhesive phenotype associated with severe malaria was well recognized by sera of malaria semi-immune children. The efficiency of the Duffy binding-like 1α (DBL1α) domain of this PfEMP1 was therefore, alone or in combination with two additional DBL1α domains, evaluated as a potential vaccine candidate using both a rodent model and a primate model. Antibodies against the DBL1α domain were generated by immunization with recombinant DBL1α-Semliki Forest virus particles and recombinant protein and analyzed in vitro. The immunized animals were challenged in vivo with various parasite strains or clones. Immunization with the PfEMP1-DBL1α domain abolished the PfEMP1-dependent sequestration of the homologous strain in immunized rats and substantially inhibited parasite adhesion in immunized monkeys. Protection against sequestration of heterologous parasite strains was also confirmed by direct or indirect challenge in the rat model. These results strongly support the use of the DBL1α domain in the development of a vaccine targeting severe malaria.

The human malaria parasite *Plasmodium falciparum* is responsible for the death of 1.5 to 2 million individuals per year, affecting mainly children under the age of 5 years (36). An effective vaccine is urgently needed and would offer one of the most promising long-term solutions in the combat against malaria.

Cerebral malaria accounts for more than one-third of the severe cases in African countries (21, 22). The primary cause of cerebral malaria is the sequestration of infected erythrocytes (iRBC) in the microvasculature of the brain (22) leading to severe endothelial damage as frequently observed in postmortem examination of patients (35, 37). Molecules or antibodies able to block the interaction between parasite ligands and human receptors that would provide therapeutic or preventive treatment are still not available.

Parasites infecting children express different variants of variable surface antigens leading to either mild or severe disease in the host. Antigens associated with severe disease are frequently recognized by sera from semi-immune individuals with various exposures to the parasite indicating a strong association between immune recognition of this virulent subtype of antigens and immunity to clinical disease (4, 6, 8, 23, 24, 39). Antibodies recognizing these surface antigens lead to a selection against the parasites expressing them (6), suggesting immunity develops first against variants associated with virulence and severe disease, while an incomplete repertoire of these specific antibodies makes the individual susceptible to severe disease (6, 8, 23, 24, 39). The fact that *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) variants of the severe subtype tend to be more immunogenic and to be better recognized than those of the uncomplicated subtype proposes that these PfEMP1 molecules are promising vaccine candidates potentially able to generate protective immunity against severe disease.

The family of PfEMP1 is so far the only group of surface antigens linked to the parasites' ability to cytoadhere and sequester (2, 14, 26). PfEMP1 is a clonally variant antigen responsible for the antigenic variation at the iRBC surface (12, 34), with an extracellular part composed of various domains. The Duffy binding-like domain 1α (DBL1α) has the highest degree of sequence conservation among all PfEMP1 domains (18) and is an attractive candidate for the development of an anti-severe malaria vaccine. Extensive analysis of the role of PfEMP1 during sequestration has revealed the importance of this domain for binding to different host receptors on RBC and endothelial cells (13, 31, 40) and its role in parasite sequestration in the microvasculature (14, 26, 40). These interactions have been linked to severe disease (9, 19, 25, 30), and immune

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TABLE 1. Scheme of animal immunization

Exptl. setup of group	Antigen used for vaccination	No. of animals	No. of vaccinations		Dose per vaccination	
			rSFV particles	Protein	rSFV particles (no.)	Protein (μg)
Rats						
Group 1 (1 DBL1α construct)	DBL1α *var1* FCR3S1.2	8	3	1	1×10^8	100
Group 2 (3 DBL1α constructs, mixed)	DBL1α *var1* FCR3S1.2 DBL1α *var1* PAvarO DBL1α *var5.2* 3D7	10	3	1	1×10^8	33 per antigen
Group 3 (3 DBL1α constructs, sequentially)	DBL1α *var1* FCR3S1.2 DBL1α *var1* PAvarO DBL1α *var5.2* 3D7	6	3	1	0.33×10^8 per antigen	33 per antigen
Group 4 (1 DBL1α construct)	DBL1α *var1* PAvarO	8	3	1	1×10^8	100
Group 5 (control)	GST	12	3	1	1×10^8	100
Monkeys						
Group A (1 DBL1α construct)	DBL1α *var1* FCR3S1.2	5	3	1	5×10^8	50
Group B (control)	GST	5	3	1	5×10^8	50

responses to this PfEMP1 domain can be important for protection against severe and complicated malaria.

We have recently demonstrated that immunization with recombinant Semliki-Forest virus (SFV) particles encoding the DBL1α domain of a parasite with a phenotype associated with severe malaria (FCR3S1.2) generates functional and biologically active antibodies. They recognize the PfEMP1 on the iRBC surface, disrupt parasite autoagglutinates and rosettes, and block iRBC adhesion in vivo (14, 26). Here we have studied the immunogenicity of our DBL1α vaccine in a novel sequestration model employing the nonhuman primate *Macaca fascicularis*. Moreover, we have analyzed the potential of a combination of several DBL1α domains (*var1* of FCR3S1.2, *var1* of PAvarO, and *var5.2* of 3D7) for their ability to induce a broader, protective repertoire of antibodies in a small animal model. Immunization with the recombinant SFV particles encoding the DBL1α domain induced strong humoral immune responses that are cross-reactive to heterologous parasites. We additionally confirmed the specific antibodies to substantially block iRBC adhesion in the vasculature of the immunized monkeys. Our data underline the importance of including the DBL1α domain in a vaccine against severe malaria based on its ability to induce antibodies mimicking those in children protected by naturally acquired immunity.

MATERIALS AND METHODS

Parasite strains and isolates from Kenya. The *P. falciparum* laboratory clone FCR3S1.2 (17) and strains FCR3CSA (27), R29, Dd2, FCR3, PAvarO, and A4 (29) were cultivated as described previously (20). The phenotype of FCR3S1.2, PAvarO, and R29 iRBC was maintained by weekly enrichment over a Ficoll gradient (20), while FCR3CSA iRBC were frequently panned on chondroitin sulfate A (CSA) to maintain the parasite phenotype.

Children attending Kilifi District Hospital (Kilifi, Kenya) with a primary diagnosis of malaria, but not fulfilling the criteria for severe malaria according to Taylor et al. (38), were recruited for the study. If consent was obtained from the child's legal guardian, a blood sample for parasite isolation was drawn. After collection, culturing was done according to standard methods; parasites were harvested for fluorescence-activated cell sorting (FACS) analysis 24 to 28 h postinvasion (p.i.).

Recognition of parasite strains/isolates by sera from semi-immune Kenyan children. Fifty-eight sera from malarious, semi-immune children (Kilifi, Kenya; approved by the legal guardians of the children) and control pools of sera from local adults and healthy Europeans were tested for recognition of seven fresh local parasite isolates from children (p78, 80, 83, 95, 1759, 3840, and 4687) and two laboratory strains (FCR3S1.2 and A4). Trophozoite stages were tested in serum dilutions of 1:5 by FACS analysis (EPIC/XL flow cytometer; Coulter Electronics) after labeling with a fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin (Ig) antibody (dilution of 1:100; The Binding Site Limited, England) and 10 μg/ml ethidium bromide, counting 1,000 iRBC.

All experiments followed the guidelines of conduct of clinical research and were performed after informed consent of the children's parents or guardians. Ethical clearance was granted by the Kenya National Ethical Review Committee.

Production of recombinant SFV particles and recombinant proteins in *Escherichia coli*. Three mini-*var* genes were created (for oligonucleotides, see Table S1 in the supplemental material) combining the open reading frames of three DBL1α domains with the transmembrane (TM) domain of the FCR3S1.2 *var1* PfEMP1 as described before (14): (i) DBL1α domain of FCR3S1.2 *var1* PfEMP1 (11); (ii) DBL1α domain of PAvarO *var1* PfEMP1 (32); and (iii) DBL1α domain encoded by *var5.2* of the 3D7 *P. falciparum* clone (chromosome 5; PfE1640) (41). Recombinant SFV particles were produced as described previously (14).

The DBL1α domains were in addition expressed as glutathione *S*-transferase (GST) fusion proteins (vector pGEX-4T-1; AmershamPharmacia, Sweden) or His-tagged fusion proteins (vector pQE-TriSystem; QIAGEN) and purified as described previously (13).

Immunization of animals. Fifty male Sprague-Dawley rats (3 months old) were assigned to five different groups (Table 1). Animals received three immunizations with recombinant SFV particles on days 0, 30, and 60 (1×10^8 particles/rat) and one immunization with recombinant protein emulsified in Montanide ISO 720 (Seppic, France) on day 90 (100 μg protein/rat) subcutaneously. Sera were collected 4 weeks after the last immunization. Animals were challenged with parasites between days 125 and 135 (permission no. N176/03).

Ten female cynomolgus macaques (*Macaca fascicularis*, 4 to 5 years old) were housed at the Primate Research Center of the Swedish Institute for Infectious Disease Control (permission no. N222/04 to N308/04). They were randomly assigned into two groups (Table 1) and immunized three times by subcutaneous administration of recombinant SFV particles on days 0, 28, and 56 (5×10^8 particles/animal) and once with recombinant protein (50 μg protein/animal) emulsified in Montanide ISO 720 on day 84. Blood samples were collected on days 56, 84, and 105; all monkeys were challenged with iRBC on day 111 or 113.

Challenge of immunized rats and monkeys with iRBC. Immunized rats were challenged by intravenous infusion with various ^{99m}technetium-labeled iRBC of parasite strains according to an established protocol (14, 26). The amount of sequestered iRBC was determined by relating the count rates of the separate lungs to the whole-body count rates comparing DBL1α- and GST-immunized animals.

For indirect challenge, iRBC (1×10^7cells/rat) were, after labeling, incubated with immune rat sera at dilutions of 1:2.5 to 1:40 at 37°C for 45 min prior to injection into naïve animals. The numbers of iRBC sequestered in these animals were compared to controls, where incubation with sera from the control group was performed.

Immunized monkeys were challenged according to a recently established method (39a). Briefly, ^{99m}Tc-labeled human RBC or iRBC were injected through a catheter into the vena saphena magna (1×10^8 cells/monkey). Images were acquired for 15 min in a three-headed gamma camera starting 20 min after injection of iRBC; anatomical three-dimensional pictures were simultaneously obtained by X ray. The amount of material sequestered in the lungs was determined by overlaying the data collected with both systems. The background level of uninfected human RBC binding was determined from one naïve monkey, while the level of specific sequestration in the animals was calculated from one naïve and four control-immunized monkeys. In order to reveal the inhibitory effect on sequestration, the values were corrected for background-binding of uninfected RBC and the amounts of iRBC in the lungs of DBL1α- and GST-immunized monkeys were compared.

Immunological analysis of anti-DBL1α antibody responses in immunized monkeys. Enzyme-linked immunosorbent assays (ELISAs) were carried out as described previously (13) using sera in double dilutions (1:1,000 to 1:64.000); endpoint titers were determined comparing the reactivity of the sera against the DBL1α domain and GST.

The presence of specific antibodies against the DBL1α domain was confirmed by immunoblotting as described previously (13) using DBL1α protein (expressed by *E. coli* and transfected BHK-21 cells) or native PfEMP1 purified from iRBC membranes (dilution of sera, 1:250).

Rosette disruption assay and serum-induced agglutination assay. The ability of the immune sera to disrupt rosettes (dilutions of 1:5 to 1:100) was assayed as described previously (13) using the purified IgG fraction of a Malawian hyper-immune serum pool and a malaria-naïve Swedish serum as controls.

To investigate immune agglutination of iRBC, trophozoite iRBC (20 h p.i.) were treated with phosphate-buffered saline containing 100 mM sodium citrate, washed, resuspended to a hematocrit of 10%, and incubated with the immune sera (dilutions of 1:50 to 1:100), and agglutinates were scored as described previously (16).

RESULTS

FCR3S1.2 is recognized by antibodies of semi-immune Kenyan children. FACS analysis of surface immunofluorescence with 58 individual sera from semi-immune children, a pool of sera from local adults and European individuals was created to investigate whether FCR3S1.2 *var1* PfEMP1 was recognized by antibodies from children in areas of malaria endemicity. In addition, 7 fresh isolates from local children with uncomplicated malaria (see Materials and Methods) and the laboratory strain A4 were tested as references. All parasites were recognized by the pool of local adult sera (54% average reactivity; data not shown) but poorly by the pool of European sera (3% average reactivity), while reactivity with the different sera from local children fluctuated considerably; on average 18%, varying between 7 and 36% for the different local isolates and parasite strains (Fig. 1). FCR3S1.2 iRBC organisms were among the best recognized parasites by the local adult pool (67% reactivity; data not shown) and the absolute best recognized parasite by sera from local children (36% average reactivity), better recognized than any of the local parasite isolates (Fig. 1). This suggests that the antigen FCR3S1.2 *var1* PfEMP1 displays strong immunogenicity and indicates the FCR3S1.2 *var1* PfEMP1 type to be commonly expressed by wild-type parasites.

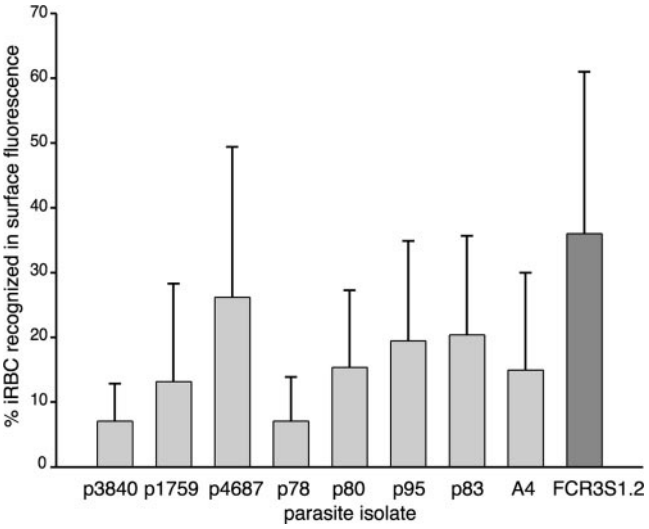

FIG. 1. Recognition of laboratory strains and local Kenyan isolates by sera from local children as analyzed by FACS. Fifty-eight sera obtained from semi-immune children in Kenya were tested for their ability to react with the surface of seven local parasite isolates and two laboratory parasite strains. The laboratory strain FCR3S1.2 (dark gray bar) shows a higher average percentage of surface fluorescence when incubated with sera from local children than the laboratory strain A4 or any of the local parasites. All parasites tested were well recognized by a pool of sera from local adults (positive control) and poorly recognized by a pool of European sera (negative control; data not shown).

The high level of antibody recognition already present in childhood supports the idea of FCR3S1.2 iRBC being a parasite with a phenotype possibly associated with severe disease.

Anti-DBL1α antibodies protect against sequestration upon parasite challenge in the rat model. Our earlier studies have demonstrated anti-DBL1α domain antibodies to be protective against challenges with homologous parasites in vivo (14). To investigate whether DBL1α domains are able to induce cross-protective antibodies, three DBL1α domains derived from different parasite clones/strains were selected as immunogens; the most importance was attached to the DBL1α domain (of *var1*) of the clone FCR3S1.2, a parasite that displays a multiadhesive phenotype and forms rosettes and large autoagglutinates, which makes it a potential model of a parasite expressing a PfEMP1 associated with severe malaria—a hypothesis supported by the immunofluorescence data presented above. Two additional DBL1α domains were included in order to analyze the capacity of a composition of DBL1α domains to induce a broad range of antibodies. The DBL1α domain (*var1*) of the clone PAvarO, a parasite that has been adapted to infect *Saimiri* monkeys, which also displays a rosetting phenotype and the DBL1α domain of *var5.2* derived from the parasite clone 3D7S8 were selected. Sequence alignment of these DBL1α domains illustrates that they cluster into different groups (see Fig. S1 in the supplemental material). The sequence similarity between the DBL1α domains used in our vaccine study varies between 43 and 57% (DBL1α domain of FCR3S1.2/*var5.2*, 45%; FCR3S1.2/PAvarO, 43%; *var5.2*/PAvarO, 57%), supporting the idea that they represent different subtypes of DBL1α domains.

Animals were immunized with single (FCR3S1.2 or PAvarO)

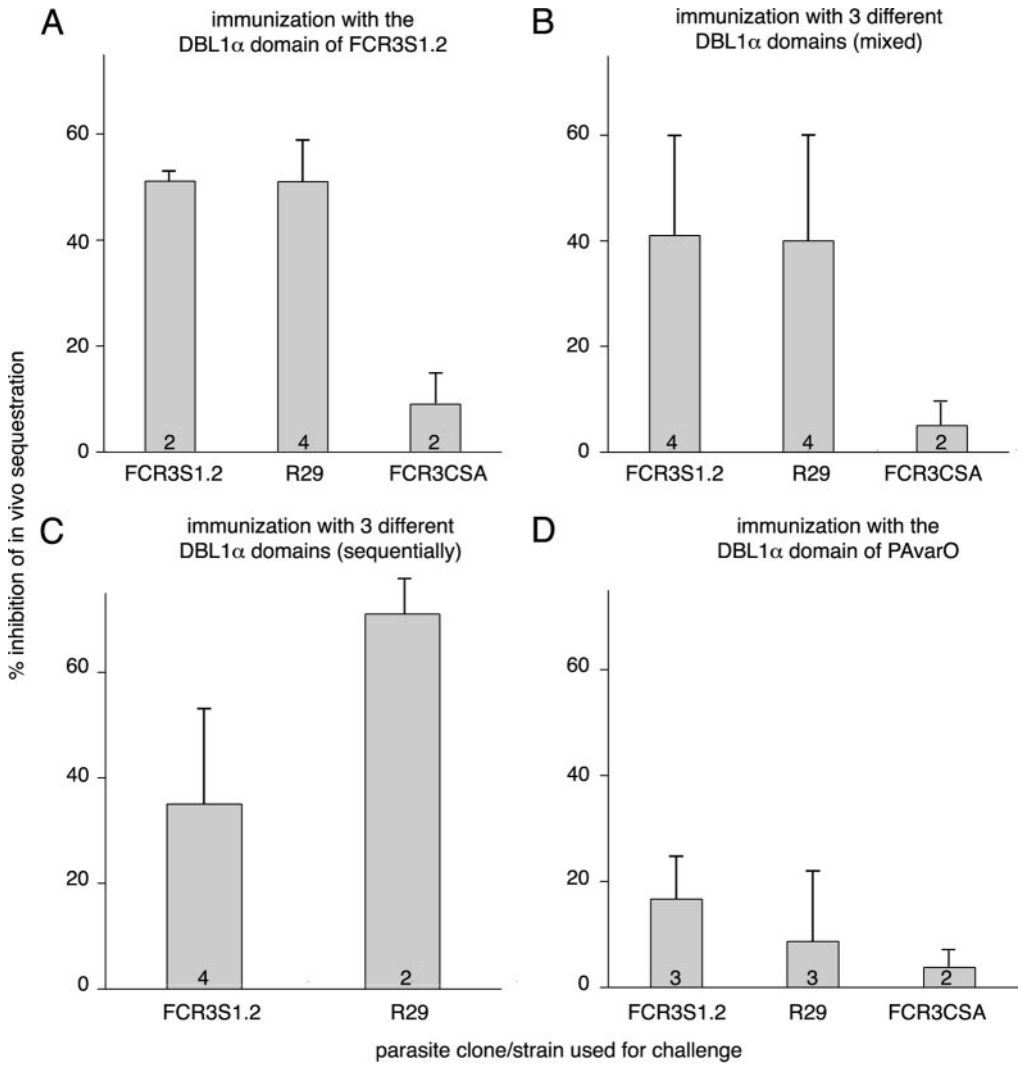

FIG. 2. Inhibition of in vivo sequestration of iRBC in immunized rats. Immunized rats were challenged with iRBC of different parasite strains/clones. Sequestration of iRBC is expressed as a percentage of that in animals immunized with a control protein. The bars give the mean values of at least two animals, and error bars are presented when more than two animals were tested. The number of tested animals is indicated in the bottom of the bars. (A) Animals were immunized with the DBL1α domain of FCR3S1.2 and challenged with iRBC of FCR3S1.2, R29, or FCR3CSA. (B) Animals were immunized with three DBL1α domains applied as a mix and challenged with iRBC of FCR3S1.2, R29, or FCR3CSA. (C) Animals were sequentially immunized with three DBL1α domains and challenged with iRBC of FCR3S1.2 and R29. (D) Animals were immunized with the DBL1α domain of PAvarO and challenged with iRBC of FCR3S1.2, R29, or FCR3CSA.

or various DBL1α domains (FCR3S1.2, PAvarO, 3D7 *var5.2*), applied sequentially or as a mixture (Table 1) followed by challenges with iRBC of different antigenic types.

Prior to parasite challenges, the presence of specific antibodies was verified. Sera obtained from groups 1 and 4 showed strong reactivity with the homologous protein in Western blots (data not shown) and ELISAs, but very weak or no cross-reactivity with the DBL1α domains derived from the heterologous *var* genes; sera obtained from groups immunized with several DBL1α domains displayed reactivity against all three domains, with a higher reactivity against the DBL1α domain of FCR3S1.2 iRBC. Differences between sera of group 2 (sequential application) and 3 (mixed application) were small, with slightly higher reactivity induced in animals of group 2 (see Fig. S2 in the supplemental material).

Surface fluorescence with the generated sera confirmed the presence of antibodies able to react with the surface of the live iRBC. Sera from all groups were tested for their reactivity with different laboratory strains/clones (FCR3S1.2, R29, FCR3, F32, Dd2, and FCR3CSA). Fluorescence was strongest using the homologous parasite FCR3S1.2, but also present as cross-reactive antibodies against the heterologous parasite strains R29 and FCR3, while Dd2 and FCR3CSA did not show any reactivity (see Table S2 in the supplemental material).

Rats from all groups were challenged with various parasite strains/clones with similar absolute sequestration levels (FCR3S1.2, 6%; FCR3CSA, 4.5%; and R29, 4.5%). The level of sequestered iRBC in the lungs was compared to that of animals immunized with a control antigen (arbitrarily set to 100%) and corrected for uninfected human RBC background

values (26). In addition, levels of sequestration of iRBC in GST-immunized and naïve animals were compared and no differences in binding levels could be observed (data not shown).

Rats immunized with the DBL1α domain of FCR3S1.2 alone (group 1) showed a mean reduction of 51% sequestrated material upon challenge with both the homologous FCR3S1.2 and the heterologous R29 parasite as compared to that of control animals (Fig. 2A). Similar results were obtained in group 2 (reduction with 41% and 40% sequestrated material; Fig. 2B) and group 3 (reduction with 34% and 71% sequestrated material; Fig. 2C) when challenged with iRBC of FCR3S1.2 and R29. Almost no reduction was observed upon challenge with FCR3CSA iRBC in any of the groups (Fig. 2A, B, and D). Rats immunized with the DBL1α domain of PAvarO alone (group 4) did not show any reduction of sequestration with any of the three different parasite clones (Fig. 2D).

Sequestration levels of iRBC lacking PfEMP1 on their surface are around 45% lower than that of iRBC with PfEMP1 (26), indicating that this remaining binding is PfEMP1 independent, possibly mediated by other surface antigens or passive trapping, revealing our generated anti-DBL1α-antibodies almost completely block the PfEMP1-dependent sequestration of iRBC.

In addition, we investigated whether the protective effect of the anti-DBL1α-antibodies could be passively transferred from immunized to naïve animals: iRBC were preincubated in serially diluted immune sera obtained from immunized rats prior to injection into naïve rats, and binding levels were compared to those of iRBC incubated in control sera, revealing a dilution-dependent inhibitory effect on sequestration of FCR3S1.2 and R29 iRBC (see Fig. S3 in the supplemental material).

Anti-DBL1α antibodies induced by vaccination in monkeys. Ten monkeys assigned to two different groups were immunized with the DBL1α domain of FCR3S1.2 or GST, respectively. Antibody responses against the DBL1α domain were measured by ELISAs and Western blotting: ELISA endpoint titers ranged between 1:32,000 and 1:1,000 (Fig. 3A). Sera of all DBL1α domain-immunized monkeys reacted with recombinant DBL1α protein and recognized PfEMP1 purified from FCR3S1.2 iRBC membranes in immunoblot assays (Fig. 3B and C).

Functional analysis of the antibodies revealed their ability to disrupt rosettes and to agglutinate FCR3S1.2 iRBC. While the rosetting rates were on average reduced with ≈11% (dilution, 1:20), 38% (dilution, 1:10) and ≈40% (dilution, 1:5) with sera of the DBL1α-immunized group, the efficiencies were 7% (dilution, 1:5), 4% (dilution, 1:10), and 3% (dilution, 1:20) for those of the control group (Fig. 4). The same sera showed no influence on the rosetting capacity of PAvarO iRBC, while sera raised against the DBL1α domain of PAvarO indeed disrupted the rosettes of this parasite (data not shown). Sera of all monkeys immunized with the DBL1α construct agglutinated FCR3S1.2 iRBC (dilution from 1:50 to 1:80), while sera of the control group did not (data not shown).

Anti-DBL1α antibodies protect monkeys against iRBC sequestration. We have recently advanced a small animal model visualizing sequestration of iRBC in cynomolgus macaques (39a). Briefly, to investigate the binding of infected and noninfected human RBC in the circulation of monkeys, two naïve

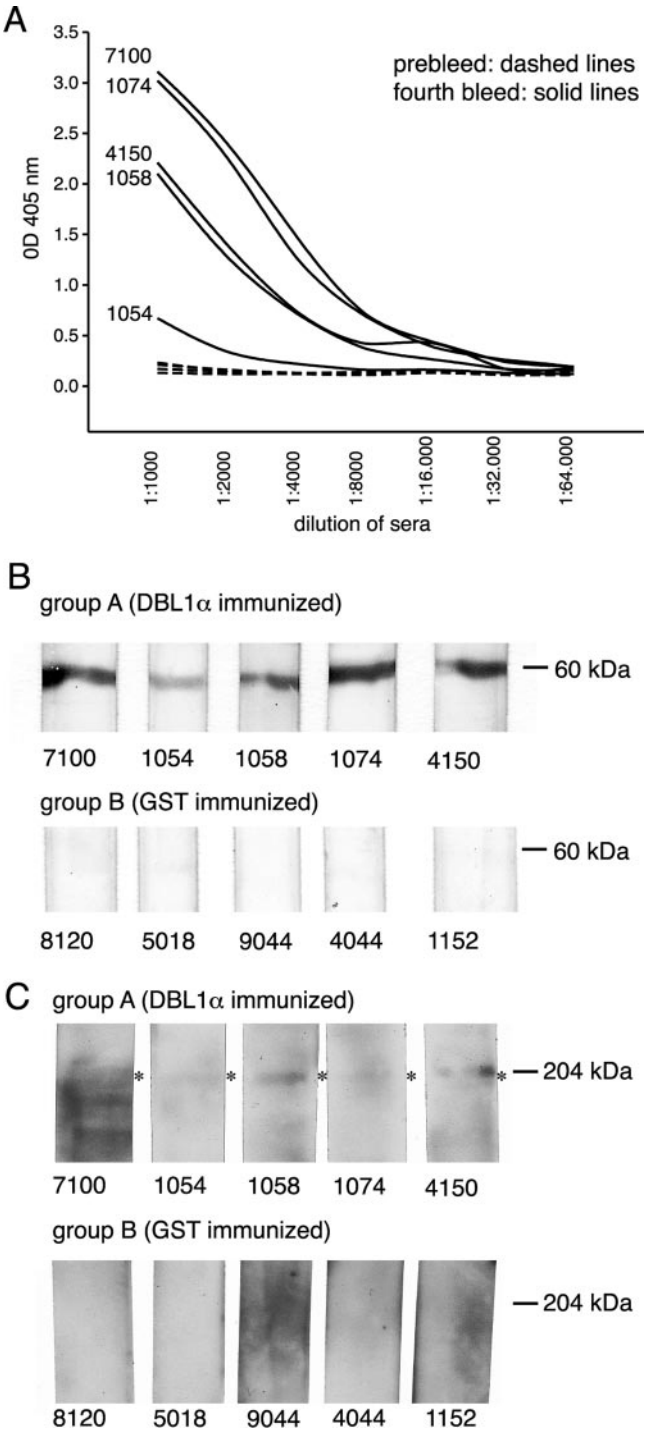

FIG. 3. Reactivity of sera generated against the DBL1α domain of FCR3S1.2 in monkeys. (A) The reactivity of the sera against a His-tagged recombinant DBL1α protein was measured by ELISA. Sera obtained from DBL1α-immunized monkeys specifically reacted with the recombinant protein (solid lines), while the prebleed sera did not (dashed line). Sera obtained from GST-immunized monkeys did not show any reactivity with the recombinant DBL1α protein in either the prebleed or the last bleed sera (data not shown). (B) Immunoblot against recombinant DBL1α protein obtained from electroporated BHK cells. (C) Immunoblot against PfEMP1 purified from iRBC membranes visualizing the reactivity to the native protein.

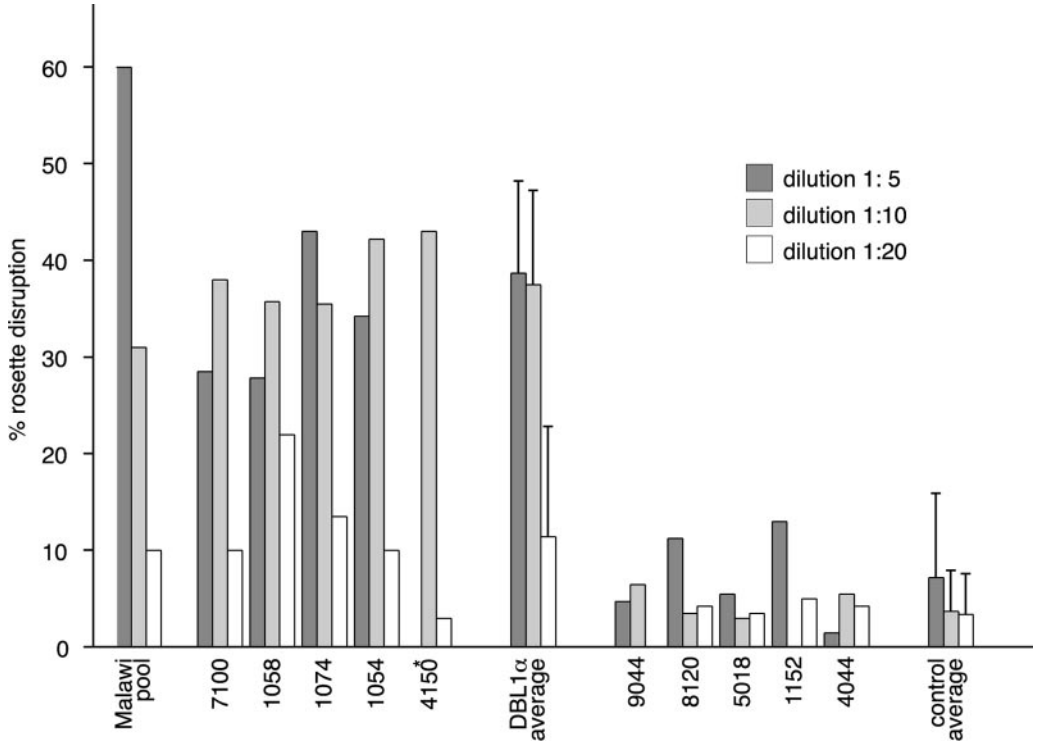

FIG. 4. Rosette disruption by immune sera generated in monkeys. The capacity of the immune sera to disrupt rosettes of the parasite FCR3S1.2 was tested: sera of monkeys immunized with the DBL1α domain showed a significantly higher rosette disruption activity than those of monkeys immunized with a control protein GST. Bars represent the means of two experiments: dark gray bars, serum dilution of 1:5; light gray bars, serum dilution of 1:10; white bars, serum dilution of 1:20. An asterisk indicates that serum of animal 4150 could not be assayed in dilutions below 1:10 due to unspecific agglutination of RBC.

animals were challenged with 1×10^8 either infected or noninfected human RBC and the allocation of the cells was observed for 60 min. iRBC revealed a binding pattern resembling that of human iRBC in the previously described rat model, with around 9% of the infected material sequestering in the lungs. Injection of noninfected RBC illustrated a background binding of human RBC in the lung endothelium of 3.4% (Fig. 5A).

To investigate the effect of our vaccine, all 10 immunized monkeys were challenged with FCR3S1.2 iRBC. Analysis of the lung endothelium revealed that immunization with the DBL1α construct substantially lowered the number of sequestered iRBC. The accumulation of iRBC in animals immunized with GST was identical to that of the nonimmunized animal during the pilot experiment. Control animals had 9.6% of the iRBC sequestered in the lungs 30 min after injection, corresponding well to 8.7% of the iRBC found in the pilot experiment 60 min after injection (Fig. 5A), considering the slow decrease of the sequestered material during the experiment as described for the rat model (26) (Fig. 5A). In contrast, monkeys immunized with the DBL1α construct had only 6.7% of the material bound in the lung capillaries. The binding in the DBL1α-immunized monkeys was therefore lowered to 54% on average compared to that of the control animals (Fig. 5B), when taking into consideration the unspecific activity of human RBC, which accounts for 3.4% of the material trapped in the lungs.

Differences in the sequestration rates between the groups were assessed with the unpaired *t* test using Sigma Stat software (SPSS, Chicago, IL); a *P* value less than 0.05 was considered significant. Comparison of the two groups revealed that the sequestration levels in the DBL1α-immunized group were statistically lower than that in the control group ($P < 0.001$), which was likewise the case for sequestration rates including or corrected for RBC background binding.

DISCUSSION

The lack of antibodies to surface antigens on *P. falciparum* iRBC associated with severe disease has been proposed as the reason why children younger than 5 years of age are most vulnerable to severe malaria (5–7, 10, 23, 24). The induction of an early immune response against this subtype of PfEMP1 molecules in those individuals would be essential to prevent severe disease.

PfEMP1 molecules mediating the sequestration of the iRBC are among the most promising candidates for an anti-severe malaria vaccine. Immune responses to this antigen are crucial for the protection against severe disease, though additional surface antigens might be involved in protection (1, 5, 7, 10, 23, 24). A potential vaccine candidate is the PfEMP1 of the parasite clone FCR3S1.2 studied here, which is able to bind to multiple host receptors (13, 40), analyzed extensively in our in vivo sequestration model based on immunocompetent Sprague-Dawley rats. Histopathological analysis of the sites of sequestration in the animal model revealed degradation of

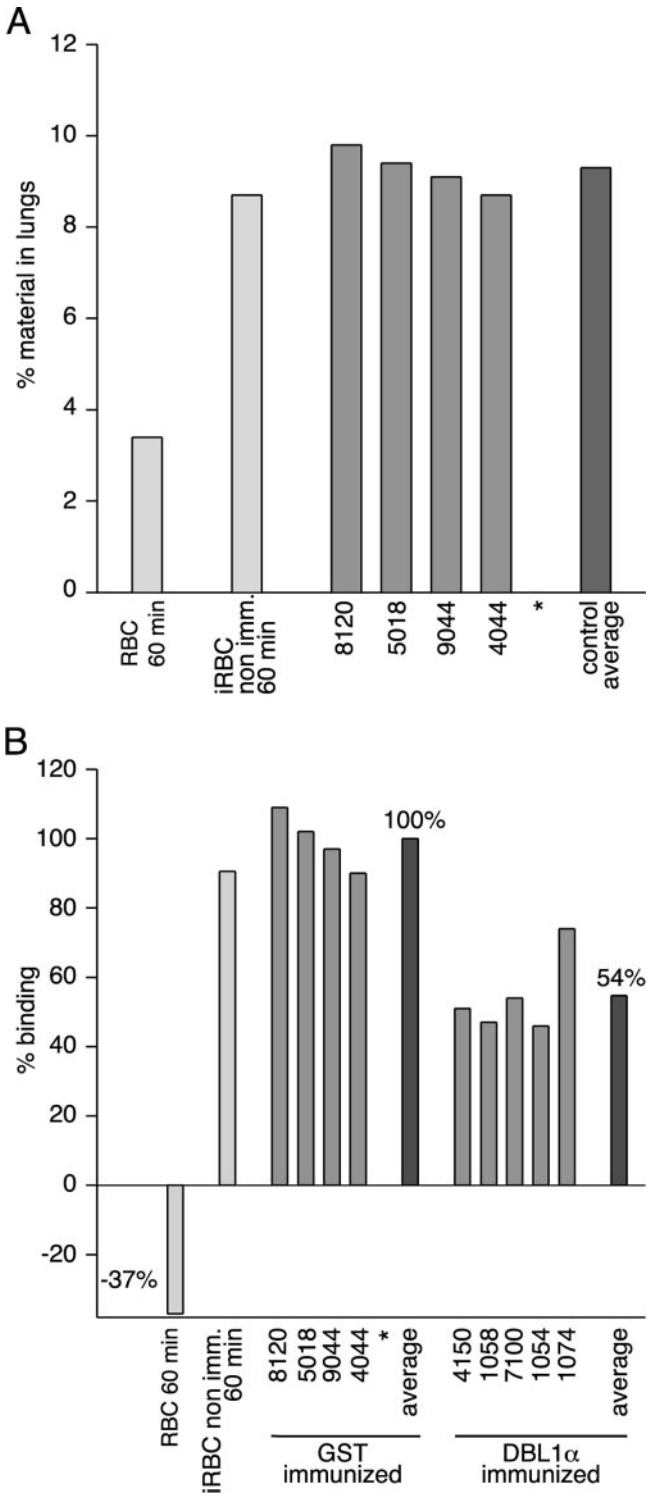

FIG. 5. Inhibition of in vivo sequestration of iRBC in immunized monkeys. (A) Sequestration of *P. falciparum* iRBC in macaque monkeys. Two naive monkeys were employed to adapt the rat model to monkeys: step 1 involved injection with uninfected human RBC in order to control for the unspecific binding of human RBC (first bar), and step 2 involved injection of iRBC into a naive animal to verify sequestration of iRBC in the monkey (second bar). In addition, four monkeys immunized with GST were challenged with iRBC of the parasite clone FCR3S1.2 and compared to binding in the naive animal to define a baseline binding of the parasite FCR3S1.2 in this model. The bars correspond to the percentage of the infected material sequestrated in the lungs. Gray bars represent individual animals of group B, and dark gray bars represent the average of the group. (B) Sequestration in immunized monkeys. The five animals belonging to group A were challenged with the same parasite in order to study the effect of the DBL1α immunization; the values are corrected for unspecific binding (first bar) caused by passive trapping of human RBC as observed in the pilot experiment. The average binding of iRBC in the monkeys of the control group B is defined as 100%, and the binding in the monkeys of group A is related to that. Light bars represent binding in individual animals, and dark gray bars represent the average value of each group. An asterisk indicates that animal 1152 of group B could not be included due to technical problems.

iRBC together with the deposition of fibrin-like material resembling the situation often observed in autopsy material from patients with severe malaria (26). The PfEMP1 of the FCR3S1.2 parasite clone is dominantly recognized by immune sera from semi-immune individuals (Fig. 1), and the adhesive features have been characterized in great detail, identifying the DBL1α domain as an important ligand for binding to host cell receptors (13). Furthermore, a separate study with more than 20 isolates from Ugandan children with severe or uncomplicated malaria revealed that immune sera raised against the DBL1α domain of FCR3S1.2 preferentially recognize parasites from severe malaria patients (Normark et al., submitted for publication). The high immunorecognition of the PfEMP1 of FCR3S1.2 supports the finding that virulence-associated PfEMP1 factors are at least semiconserved (24) and that early development of immunity to these virulence factors would provide protection against lethal malaria infection.

We have previously shown that immunization with the DBL1α domain can generate biologically functional antibodies able to disrupt rosettes and parasite agglutinates and block iRBC adhesion in a rat model (14). Here, we have further investigated the cross-recognition of these antibodies generated by immunization with either a single DBL1α domain or with three DBL1α domains selected from phylogenetically distant PfEMP1. Sequential or mixed immunization with three different DBL1α domains did not generate stronger cross-protective antibodies compared to immunization with the single antigen. These data indicate that the cross-boosting effect between DBL1α domains of different PfEMP1 types is weak and underline why immunity to malaria requires numerous clinical episodes to develop.

Antibodies generated against the FCR3S1.2 DBL1α domain blocked adhesion of both FCR3S1.2 and R29 iRBC with similar efficiency (Fig. 2A), while weak inhibition of sequestration was seen in animals immunized with the PAvarO DBL1α domain regardless of the parasite strain used for the challenge (Fig. 2D). The similarity between the domains of FCR3S1.2 and R29 or PAvarO is 50% or 42%, respectively. Although the sequence identity between the DBL1α domain of the parasites R29 and PAvarO is 68%, the PAvarO construct induced no protection against challenge with R29 iRBC, indicating that despite the relatively large sequence similarity, the capacity to induce cross-reactive antibodies varies greatly (32).

Furthermore, a comparison of the sequences used for immunization here reveals a divergence between the DBL1x do-

main of FCR3 *var*2 CSA and the other domains used for immunization (sequence similarity to DBL1α FCR3S1.2, 36%; sequence similarity to DBL1α PAvarO, 37%), explaining the low level of protection against sequestration of the FCR3CSA iRBC. This is in line with previous findings that the cross-immunorecognition between parasites of cerebral and placental malaria is very limited. These parasites are associated with different adhesive and antigenic properties, given that placental parasites express a molecularly very distinct type of PfEMP1 (3, 28, 33).

Various studies based on DNA or RNA vaccination have revealed that the immunogenicity of vaccine candidates visualized in rodent models is often not reproducible in primates or humans (reviewed in reference 15). To prove the immunization regimen of SFV DBL1α particles and recombinant protein to generate functional antibody responses in primates, macaque monkeys were immunized according to our established protocol. The immunizations did not induce any adverse effect in any of the animals, indicating that the antigen itself and the adjuvant in the SFV system are safe.

The injection of infected and uninfected human RBC into the circulation of monkeys revealed around 9% of the material sequestering in the lungs, additional binding was seen in the bone marrow of the vertebrae, humerus, and hips (39a), while uninfected human RBC were detected in the heart, indicating that these cells stay in circulation. This novel primate model opens the way to study sequestration of iRBC in a physiological setting close to humans. Although artificial, it offers the possibility of investigating the effect of a vaccine aiming at the prevention of sequestration under flow dynamics and in the presence of nonmanipulated endothelial cells (26) creating valuable data that would complete the information gained from our in vitro experiments. The anti-DBL1α antibodies blocked iRBC adhesion in four out of five immunized monkeys, while the anti-GST antibodies did not show any inhibitory effect (Fig. 5). Interestingly, in another set of experiments studying desequestration of iRBC, up to 60% of the sequestered iRBC were readily released from their binding sites after administration of depolymerized heparin into the circulation (39a), arguing that the binding between the DBL1α domain and heparan sulfate (26) is one important mechanism also for iRBC binding in this model.

In conclusion, immunization with the PfEMP1-DBL1α domain of a parasite with a phenotype associated with severe malaria generates protective immunity, as demonstrated in a rodent and a primate model as well as in in vitro assays. The presence of anti-DBL1α antibodies reduced the sequestration of iRBC as efficiently as the removal of the PfEMP1 from the iRBC surface. This implies that the PfEMP1-dependent sequestration can to a large extent be completely blocked by the antibodies induced by the DBL1α domain. The FCR3S1.2 PfEMP1 is as recognized as antigens of other parasites expressing variant surface antigens associated with severe disease by immune sera of both children and adults from areas of malaria endemicity. The anti-DBL1α antibodies not only blocked adhesion of parasites with rosetting phenotypes similar to the homologous parasite but also preferentially recognized PfEMP1 expressed by parasites from children with severe malaria. These data support further studies on this promising antigen for the development of an anti-severe malaria vaccine.

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